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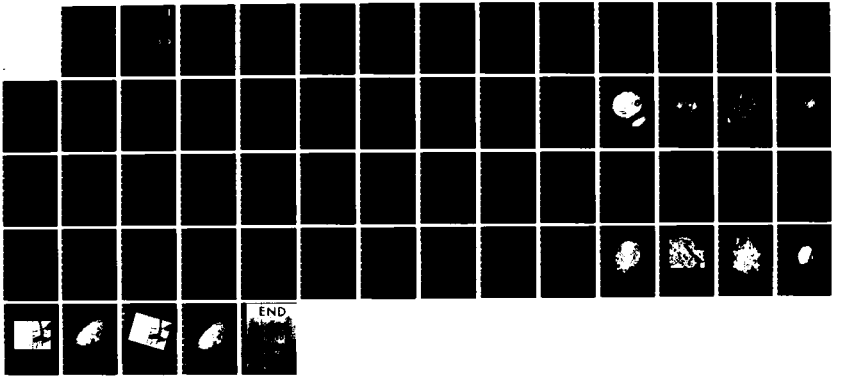

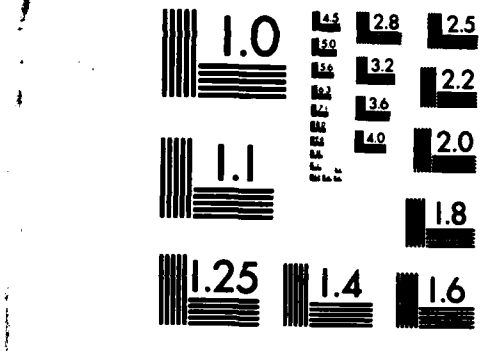

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Annual Report #1

ELECTRON MICROSCOPY OF INTRACELLULAR PROTOZOA

Masamichi Aikawa, M. D.
August, 1980

Supported by

U. S. Army Medical Research and Development
Command, Fort Detrick, Frederick,
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The Institute of Pathology, Case Western Reserve University
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During this fiscal year, in collaboration with Dr. Diggs and his associates of WRAIR, we studied a) inhibitory effects of immune monkey serum on synchronized P. falciparum cultures and b) antibody-dependent phagocytosis of Trypanosoma rhodesiense by murine macrophages.

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Studies on phagocytosis of Trypanosoma rhodesiense by macrophages showed that macrophages can bind and ingest the parasites in the presence of specific antibody. The complement system participates in the association of macrophages and T. rhodesiense, although antibody-dependent binding of trypanosomes to macrophages was also observed in the presence of antiserum depleted of complement by heating at 53°C for 90 minutes. However, when lower, less effective concentrations of antiserum were utilized, the addition of complement profoundly amplified the association of trypanosomes and macrophages. Electron microscopy revealed many trypanosomes within vacuoles of of macrophages in the presence of antiserum. Incubation of macrophages with trypanosomes and immune serum for 90 minutes resulted in no morphological changes. However, by 120 minutes, the parasites had become degenerated. No phagocytosis of T. rhodesiense by macrophages occurred in the presence of normal serum.

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Summary

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Foreward

In conducting the research described in this report, the investigators adhered to the Guide for Laboratory Animal Facilities and Care , as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Science - National Research Council.

Detailed Report

a) Inhibitory Effects of Immune Monkey Serum on Synchronized *Plasmodium falciparum* Cultures.

b) Antibody - Dependent phagocytosis of *Trypanosoma rhodesiense* by muring macrophages.

Table of Contents

Inhibitory Effects of Immune Monkey Serum on Synchronized *Plasmodium falciparum* Cultures*

Jeffrey D. Chulay, Masamichi Aikawa,

Carter Diggs and J. David Haynes

Department of Immunology, Walter Reed Army Institute of Research, Washington, D. C. 20012, and Institute of Pathology, Case Western Reserve University, Cleveland, OH 44106

Studies of immunity to malaria in various host-parasite systems have demonstrated the importance of serum factors. In human (1), rodent (2), and simian (3) infections, protection against malaria can be passively transferred by immune serum. At least part of the protective effect of immune serum is due to antibody, since it is associated with the IgG fraction of serum (1,2) and can be removed by absorption with antiserum against immunoglobulins (4).

There is also evidence that non-antibody humoral factors may be involved in protection against hemoprotozoa. Clark, et al, demonstrated intracellular death of Babesia microti concurrent with a falling parasitemia in this self-limited infection (5). These abnormal intracellular parasites were felt to be analogous to the "crisis forms" of P. brasilianum in cebus monkeys described by Taliaferro and Taliaferro (6). Intracellular death of B. microti or Plasmodium vinckei can also be produced by pretreating mice with intravenous BCG or a variety of other agents which stimulate macrophages (7,8). Similar "crisis forms" have not been described for P. falciparum, perhaps because mature parasites are rarely seen in peripheral blood (9).

Using recently developed methods for the in vitro culture of P. falciparum (10,11), several investigators have demonstrated that serum from owl monkeys immune to P. falciparum infection can inhibit parasite growth in vitro (12-14). We report here the use of synchronized P. falciparum cultures to study the sites of action of such immune serum.

MATERIALS AND METHODS

The details regarding immunization of owl monkeys (Aotus trivirgatus), in vitro cultivation of P. falciparum, and assessment of inhibition of growth by immune monkey serum will be reported elsewhere (Chulay, JD, Haynes, JD, Diggs, CL, manuscript in preparation). In brief, an owl monkey (A076-049, male, karyotype III) was immunized by infection with 1×10^6 parasitized owl monkey erythrocytes

(Camp strain of P. falciparum [15]) followed by chloroquine treatment and challenge two weeks later with $1x10^7$ parasitized autologous erythrocytes. Seven months after initial infection, rechallenge with $5x10^8$ parasitized erythrocytes caused no detectable parasitemia. Serum was obtained 7 months before initial infection and one month after rechallenge, stored at -70°C, and heat inactivated (56°C, 30 minutes) immediately prior to use.

Fifty hours prior to adding test sera, a culture of the Camp strain of P. falciparum was partially synchronized by sorbitol lysis of mature forms (16). Two 5 ml cultures (parasitemia 1.7%) were pooled and centrifuged (300 x g, 5 minutes, room temperature). The packed erythrocytes were resuspended in nine volumes of 5% sorbitol in 0.01 M sodium phosphate buffer (pH 7.4), allowed to stand 5 minutes at room temperature, and recentrifuged. The packed erythrocytes were resuspended in complete culture medium (RPMI 1640 with 25 mM HEPES acid, 32 mM $NaHCO_3$, and 10% human plasma) and diluted 1:8 with washed uninfected human type O positive erythrocytes. Five ml of a 6% suspension of this culture was added to a 25 cm^2 tissue culture flask which was flushed with a mixture of 5% O_2, 5% CO_2, 90% N_2, sealed, and incubated at 37°C. Medium was aspirated and replaced with four ml of fresh medium after 20 and 44 hours.

Fifty ul of a 1:5 dilution of normal human, normal monkey, or immune monkey serum in complete culture medium with 10 ug/ml gentamycin was added to replicate wells of flat-bottom microtiter plates. The plates were placed in a gas-tight box which was flushed with the 5% O_2, 5% CO_2 gas mixture, sealed, and incubated at 37°C for several hours. The partially synchronous culture was diluted with an equal volume of complete culture medium with gentamycin, and 50 ul was added to the microtiter wells containing test sera. The plates were returned to the gas-tight box, flushed with the gas mixture, sealed, and incubated at 37°C. After 25 hours, medium was removed from one plate and 100 ul fresh medium, containing 10% test serum and 10% human plasma, was added. After 25 and 44 hours, the plates were harvested for morphological evaluation.

For light microscopy, thin smears were prepared from triplicate wells and stained with Giemsa. The number of uninfected erythrocytes and erythrocytes infected by each parasite stage were counted until 100 parasites of each stage or 5000 total erythrocytes were identified. For each condition, 100 schizonts were also scored for the number of nuclei per schizont.

For electron microscopy, the contents of seven wells were pooled for each condition, centrifuged (500 x g, 5 minutes, 4°C) and the packed cells resuspended in 10 ml ice cold fixative (1.25% glutaraldehyde in 0.1 M cacodylate buffer with 4% sucrose, pH 7.25). The pellet was dehydrated in an ascending series of ethanol and embedded in Epon 812. Sections were cut with a Dupont diamond knife with Sorvall MT-2 ultramicrotome, collected on Nickel grids, stained with 1% lead citrate and uranyl acetate, and examined with a Siemens Elmiskop 101 electron microscope.

RESULTS

The initial culture had a 2.2% parasitemia (220 parasitized erythrocytes per 10,000 erythrocytes) with 97% ring forms, 1% mature trophozoites, and 2% schizonts. In cultures containing normal human or normal monkey serum, most parasites had developed into mature trophozoites and schizonts after 25 hours (figure 1). A few of the original parasites had completed schizogony and infected new erythrocytes (ring forms at 25 hours), with a rise in total parasitemia to approximately 3.2%. By 44 hours virtually all parasites had completed schizogony, parasitemia had increased to 14%, and erythrocytes containing ring forms accounted for 82 to 96% of all parasitized cells.

A different pattern was found in cultures grown in immune monkey serum (Figure 1). After 25 hours, the number of mature trophozoites and schizonts was similar to normal serum cultures. However, total parasitemia was only 2.1%, and there were 86% fewer new young rings than in normal serum cultures. After 44 hours the differences were greater, with 91% fewer ring forms and a total parasitemia of only 1.4% in immune serum cultures.

No morphological abnormalities were detected by light microscopy in intracellular parasites grown in immune monkey serum. After 25 hours, trophozoites and schizonts were similar in number (figure 1) and normal in appearance (figure 2a-b) whether grown in normal or immune serum. There were no differences between normal and immune serum cultures in the number of nuclei per schizont (figure 3). The few ring forms found in immune serum cultures at 44 hours also appeared healthy (figure 2c). Extracellular mature parasites were observed occasionally in all cultures. The only difference noted by light microscopy was that groups of extracellular merozoites clustered around pigment (figure 2d-f) were found frequently in immune serum cultures (an average of 76 clusters per 10,000 erythrocytes). The individual merozoites in these clusters often appeared to be disintegrating, with vacuolated, poorly staining cytoplasm. Clusters of merozoites were rarely found in cultures with normal serum (figure 1).

When cultures grown with immune serum were evaluated by electron microscopy, parasites within intact erythrocytes appeared normal (figure 4). Extracellular schizonts, however, were often abnormally vacuolated with cytoplasm less electron-dense than normal and partial disruption of the plasma membrane (figure 5). These parasites were covered by a 60 nm electron-dense surface coat.

Erthrocytes infected with P. falciparum showed excrescences on the erythrocyte membrane which were 45 nm in height and 100 nm in diameter at the base (figure 4). These excrescences were more numerous on schizont-infected cells than on cells infected with uninucleate parasites. Electron-dense precipitates over the excrescences were seen in immune serum cultures after 44 hours (figure 6), but not after 25 hours (figure 4). Partially lysed parasitized erythrocytes were found occasionally in all cultures, and in the presence of immune serum the parasites within these crythrocytes had similar precipitates on their surface (figure 6).

Many free merozoites were found in immune serum cultures, often with disrupted plasma membranes and a matrix which was vacuolated and less electron-dense than normal (figure 7). The merozoites were covered with a 60 nm, loosely packed surface coat. In many areas this coat appeared to consist of two layers. Aggregates of merozoites were observed with adherence between adjacent surface coats.

DISCUSSION

Individuals who recover from malaria are at least partially resistant to reinfection (17,18), although the mechanisms involved in acquired resistance are incompletely understood. It has been suggested that antibodies directed against merozoites can inhibit reinvasion either by agglutination of merozoites (19) or by blocking surface receptors, analogous to viral neutralization (20). Miller, et al., incubated P. knowlesi-infected erythrocytes in immune rhesus monkey serum and observed aggregation of spontaneously released merozoites (19). Due to their large size, these aggregates were unable to invade new crythrocytes. Our findings with P. falciparum cultured in immune owl monkey serum are remarkably similar. There was reduced invasion into new erythrocytes associated with clustering of merozoites around malaria pigment. The aggregation of merozoites and inhibition of in vitro growth by immune serum is antibody mediated, since it can be demonstrated using isolated immune IgG (Chulay, JD; Haynes, JD; Diggs, CL, manuscript in preparation). These anti probably account for the thickened, bilayered appearance of the surface coat on merozoites in immune serum cultures, as compared with the 20nm surface coat seen on merozoites in cultures without immune serum (Aikawa, M: unpublished data). The abnormal appearance of many individual merozoites within clusters may be due to an autolytic phenomenon, since it is known that extracellular merozoites rapidly lose their viability and morphologic integrity even

in the absence of immune serum (21). The possibility of direct merozoite damage by immune serum in the absence of complement seems less likely.

Erythrocytes infected with various species of Plasmodia develop membrane excrescences (22). Kilejian, et al, demonstrated that rat antiserum, developed against P. falciparum-infected erythrocytes and absorbed with normal erythrocytes, will bind specifically to these excrescences (23). Our demonstration that electron-dense precipitates develop on erythrocyte excrescences in cultures grown with immune serum indicates that antibody to these excrescences develops during the course of infection and acquired immunity. Langreth and Reese have also shown that antibody to excrescences develops following infection or after immunization of owl monkeys with cultured parasites (24). They were unable to demonstrate lysis of infected red cells by complement and antibody to the excrescences, and it is unknown whether antibody of this specificity is capable of inhibiting parasite growth.

Low level spontaneous lysis of parasitized erythrocytes occurred in all cultures. The precipitate seen on schizonts within such cells (figure 6) is probably antibody which entered the cell after the erythrocyte membrane was disrupted.

In the presence of an intact erythrocyte membrane, we were unable to demonstrate damage to intra-erythrocyte parasites cultured in immune serum. We were unable to find anything resembling degenerate intracellular "crisis forms". However, since we used heat-inactivated serum at a 10% concentration, it is possible that heat-labile factors, factors active only at higher concentrations, or factors appearing only transiently following immunization, may contribute via other mechanisms to humoral immunity in vivo. Our data clearly supports the hypothesis that anti-merozoite antibodies agglutinate P. falciparum merozoites and thereby inhibit their ability to invade new erythrocytes.

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LEGENDS TO FIGURES

Figure 1. *In vitro* growth of *P. falciparum* in normal and immune serum. R = ring forms, T = mature trophozoites, S = schizonts, * = extracellular merozoites clustered around pigment. The bars and numbers in parentheses represent means for triplicate determinations in one experiment.

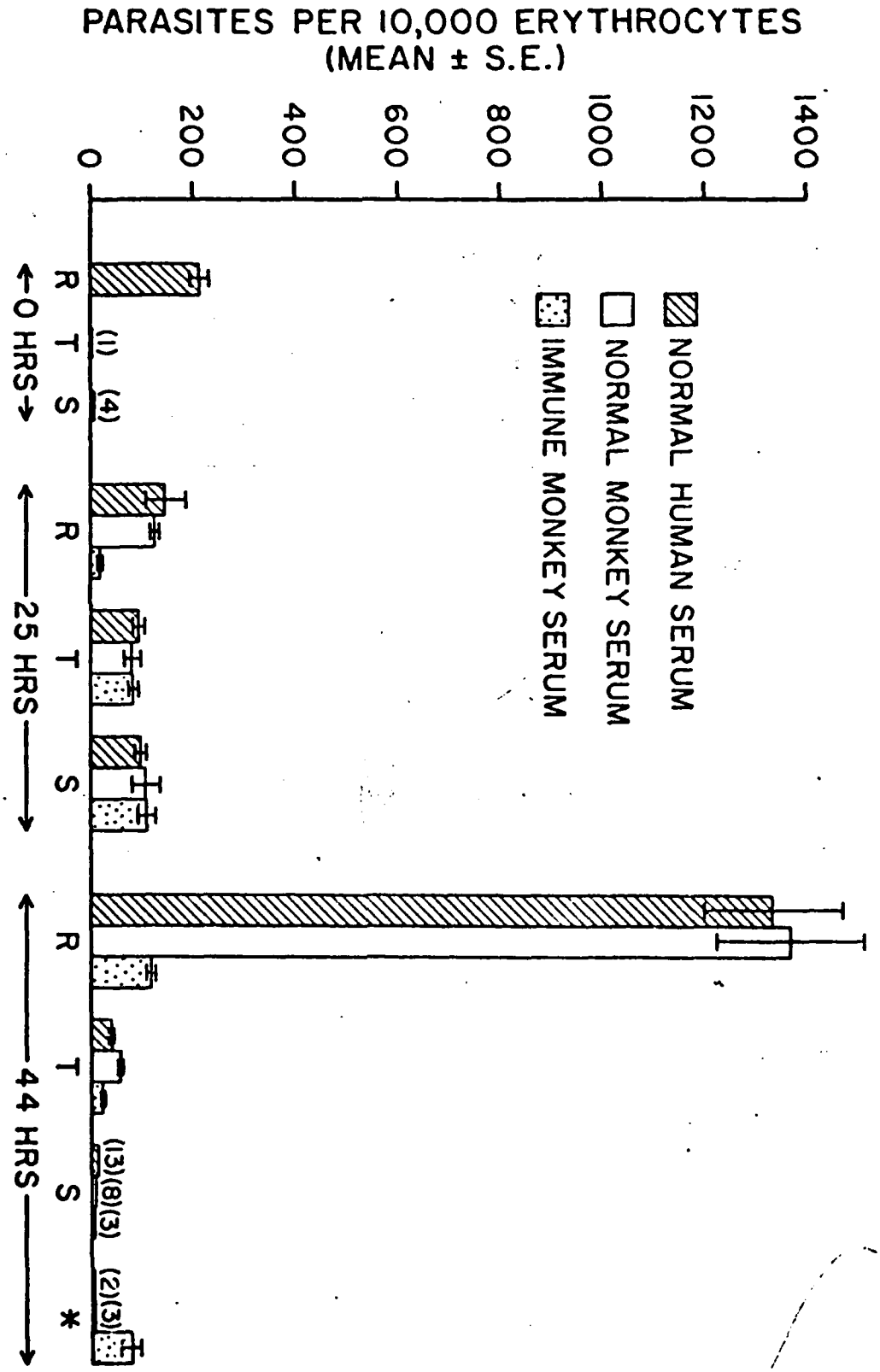
Figure 2. Cumulative distribution of the number of nuclei per schizont in parasites cultured with normal or immune serum for 25 hours.

Figure 3. Electron micrograph of an erythrocyte infected with P. falciparum schizonts (S) in immune monkey serum after 25 hours. Note food vacuoles (F) and excrescences on the erythrocyte membrane (E). x 24,000.

Figure 4. Electron micrograph of an extracellular schizont in immune monkey serum after 44 hours. The surface is covered with an electron dense surface coat (arrow). x 60,000.

Figure 5. Electron micrograph of a partially lysed erythrocyte infected with *P. falciparum* in the presence of immune monkey serum after 44 hours. Electron dense precipitates (arrow) are seen over excrescences on the erythrocyte membrane. Precipitates are also present on the parasite surface. x 30,000.

Figure 6. Electron micrograph of free merozoites aggregated in immune monkey serum after 44 hours. Note adherence (arrow) between the surface coats of adjacent merozoites. x 42,000.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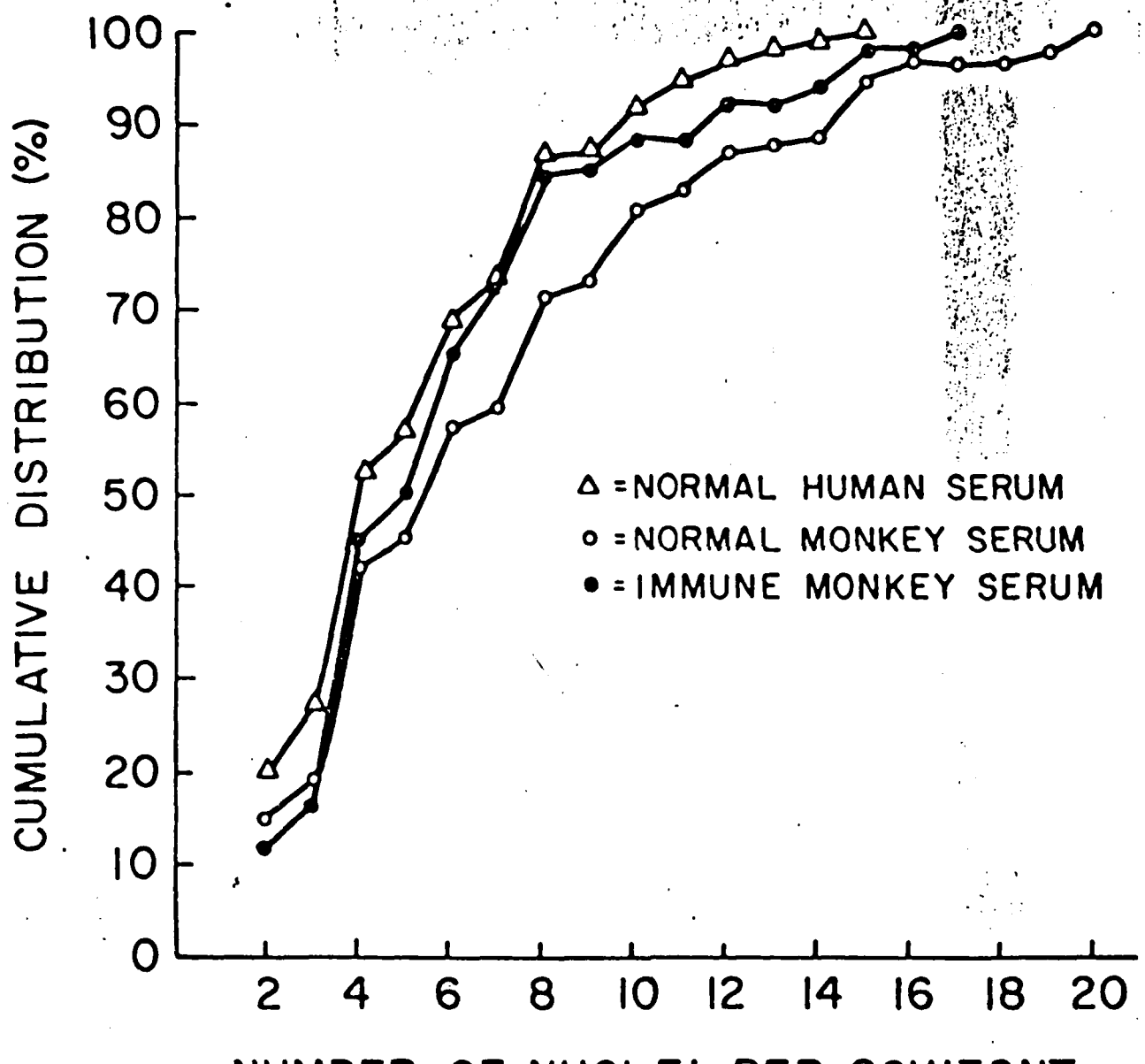
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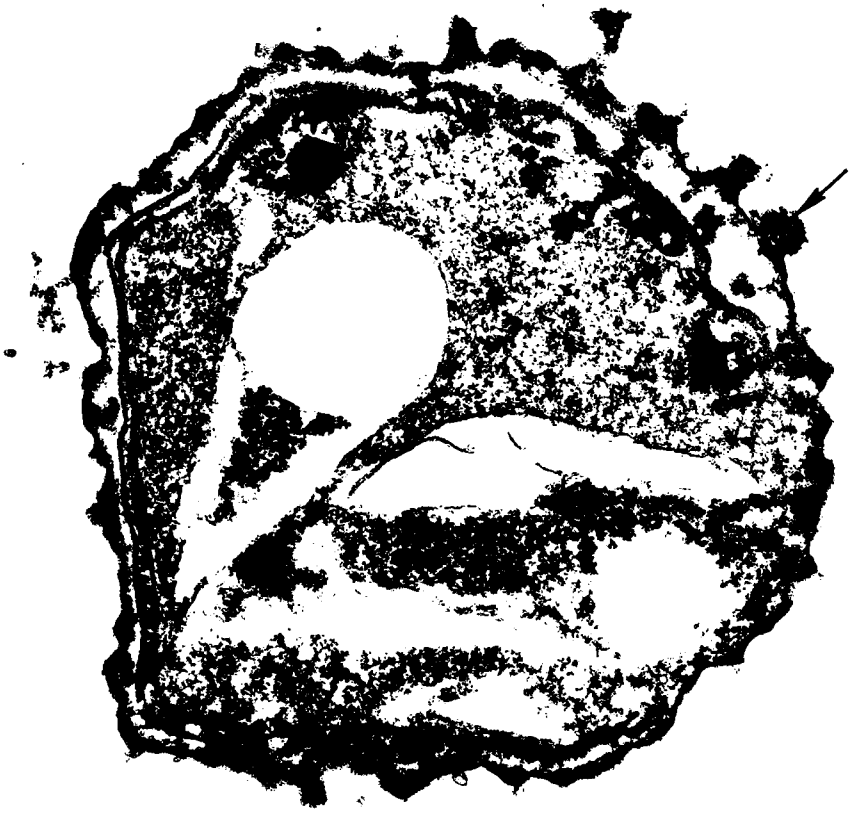

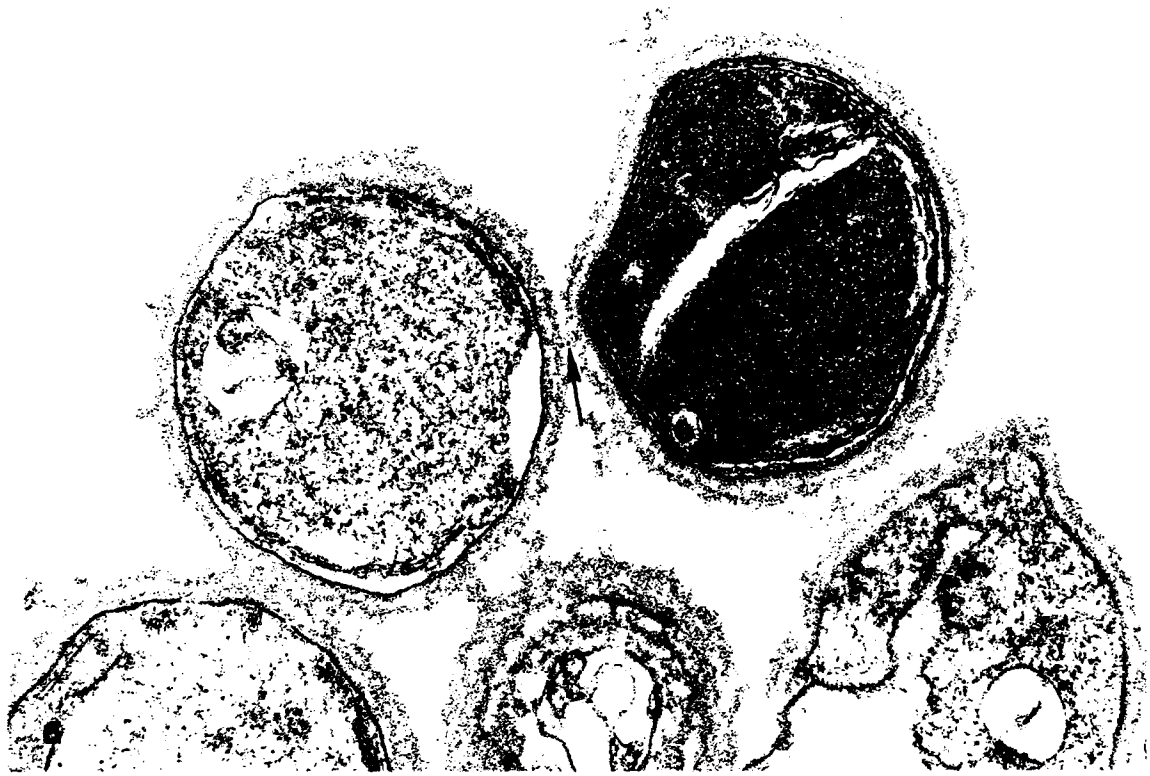

Antibody-Dependent Phagocytosis of Trypanosoma rhodesiense by Murine Macrophages.

Hellen C. Greenblatt, Carter L. Diggs, and Masamichi Aikawa

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44106

African trypanosomes present a significant human health problem and a major obstacle to agricultural development and animal husbandry (42, 63). Despite their importance, the immune responses of the host to these parasites are not adequately understood. Trypanosomiasis results in hypergammaglobulinemia (18,26,32,33), hypocomplementemia (20,26,39), circulating immune complexes (26,38,39), and immunosuppression (1,3,9, 19,36). Infection is rapidly followed by a prompt specific and non-specific antibody response (17,23,33), but antibody alone does not protect an animal from challenge (6). Efficient in vitro killing of Trypanosoma rhodesiense requires specific antibody and a functional alternative complement pathway (16). Treatment of serum with cobra venom factor (CVF) to inactivate complement, eliminates its ability to kill trypanosomes in vitro (16). However, in vivo, mice treated with cobra venom factor (CVF) are still able to effectively neutralize trypanosomes which have been sensitized with immune serum (10). Other investigators have demonstrated that T. musculi or T. lewisi infection in complement-deficient mice and rats does not differ substantially from that observed in normal animals (24). Also, lysis of T. lewisi was not observed when the parasites were placed within Millipore chambers implanted within the peritoneal civities of immune, complement-intact rats (13). It therefore appears likely that trypanosomes are not killed in vivo solely by antibody and complement, but that additional factors, perhaps cellular components, are also required.

In view of the conclusions reported by other investigators (13,15,28,54,55) and the results presented in this report, there is strong suggestive evidence

that mononuclear phagocytic cells are major agents in the elimination of antibody-sensitized trypanosomes.

Investigators have demonstrated that T. lewisi, T. brucei, and T. gambiense will bind to rat, rabbit and mouse peritoneal exudate cells respectively in the presence of specific antibody (7,8,14,28,30,50,54,55, 58,60).

Questions regarding the state of macrophage activation required for trypanosome binding, the cytophilic or opsonic nature of the antibody required, the kinetics of post-immunization generation of antibody, and the role of complement in the association of parasites and phagocytic cells were not resolved in these previous studies.

Materials and Methods:

Animals. C57BL/6J male mice were obtained from the Jackson Laboratories, Bar Harbor, Maine and used at 6-10 months of age. Mice were used as a source of peritoneal exudate cells or trypanosomes. CDF inbred male rats from Charles River Breeding Laboratories, Wilmington, Maine, were utilized either for anti-trypanosome serum production, or as a complement source.

Parasites. The Wellcome strain of Trypanosoma rhodesiense was isolated from a human in 1934, passaged through mice and rats for a number of years (45) and is currently maintained as a stabilate in our laboratories. The stabilate is stored at -70 C as a suspension in 15% (v/v) glycerol in culture medium RPMI 1640 with glutamine (Flow Laboratories, Rockville, Maryland).

Harvest of Parasites. C57BL/6J male mice or CDF rats were subjected to 900 R, 13F, Cs, gamma irradiation, (Gammacell-40, Atomic Energy of Canada Ltd.) and injected with the Trypanosoma rhodesiense stabilate. Two to 3 days post-injection the mice were killed by exposure to CO_2, their axillary vessels cut and the parasite-laden blood removed with heparinized capillary pipettes. The pooled blood was centrifuged at 1400 xg for 10 minutes at 9°C. The serum was aspirated and the trypanosome layer was removed, with care to limit contamination with erythrocytes, and added to 1 ml cold RPMI 1640. Twenty microliters of the trypanosome suspension were diluted in 2 ml of a 0.05% solution of Nile Blue sulfate (21), incubated for 5 minutes, and the trypanosomes counted in a hemocytometer. Viable parasites were suspended at the desired concentration in RPMI 1640 and held on ice until use not more than three hours later.

Preparation of antiserum against Trypanosoma rhodesiense. Except where specified, hyperimmune rat serum was used throughout. This serum was obtained by injecting CDF rats with trypanosomes which had been irradiated with 70K rads using a 60 Co gamma source (Gammacell-220, Atomic Energy of Canada, Ltd.). A total of about 10^7 irradiated trypanosomes were injected each week for a total of 5 weeks, after which the rats were bled by aortic puncture and the serum obtained. Immune serum also prepared by injecting C5FBL/6J mice or CDF rats with a single inoculum of $5x10^7$ irradiated trypanosomes. After 7d mice or rats were bled and antiserum collected. Chronic antiserum was obtained by immunization of C5FBL/6J male mice with 10^7 irradiated parasites. Serum samples were collected at 3 days and at the end of the 1st, 2nd, 4th, and 8th week after injection. A booster challenge of 10^7 irradiated trypanosomes was given to each mouse at the end of week 7.

Complement. Normal CDF rats were bled via aortic puncture. Pooled blood was permitted to clot at room temperature for 30 minutes, and placed at 9-10C for 2-3 hours. Serum was separated from the clot by centrifugation at 10,000 rpm for 10 minutes, then removed and frozen at -70 C.

Heat inactivation of complement. Complement was heated at 53 C for 90 minutes, instead of the conventional 56 C for 30 minutes to minimize immunoglobulin aggregation and development of anti-complementary activity (48,49).

Cell lines. P388D1 cells were a gift of Dr. Marta Wade, National Cancer Institute, NIH, Bethesda, Maryland. BHK21 (clone-15) was obtained from Dr. Joel Dalrymple of Walter Reed Army Institute of Research, Washington, D. C. Vero cells were provided by Dr. Robert B. Tesh, Pacific Research Section, University of Hawaii, HI.

Macrophage harvest and cultures. Normal, untreated mice were killed with CO_2, washed with 70% ethanol, and injected with 6 ml of RPMI 1640 with 50 units of heparin/ml. The abdomen was massaged, a pocket was formed in the skin, and the peritoneal cavity exposed. The peritoneal cell suspensions were aspirated with sterile pipettes and placed in polypropylene tubes (Falcon No. 2070 Oxnard, CA) in an ice bath. RPMI 1640 was injected and the cell suspensions aspirated until a total of 10 ml of cell suspension was obtained from each mouse. The cells were washed twice by centrifugation in RPMI 1640. Greater than 95% of macrophages were viable as assessed by 1% trypan blue exclusion. Cells were resuspended at the desired concentration in RPMI 1640 with 20% fetal calf serum (FCS) (Rehatuin, Reheis Chemical Co., Phoenix, Arizona), and 1% streptomycin, penicillin, and Fungizone (Microbiological Associates, Rockville, Maryland). An 0.3 ml suspension of cells at 1×10^6/ml was placed into each chamber of an eight-chamber Lab-Tek Tissue Culter Chamber Slide (Lab-Tek Products No. 4808, Naperville, Illinois), and incubated on an aluminum plate at 37C in a 5% CO_2 humidified incubator. Eighteen hours after incubation, the Lab-Tek chambers were agitated by shaking, flicked to remove non-adherent cells, and the medium replaced with warm phosphate buffered saline (PBS). After a total of 3 such washes, 0.3 ml of RPMI 1640 with 20 FCS and antibiotic were added and incubation continued for 48h more. Prior to assay, cell monolayers were washed 2-3 times with warm PBS, and the test reagents added as described below.

Assay of macrophage-trypanosome interactions. All studies were carried out directly on 48-12h adherent cell monolayers plated on Lab-Tek slides. In the

standard assay, immune and normal sera were adjusted with RPMI 1640 to final concentrations as noted in the figure legends. Serum, 0.15 ml, was placed into each chamber followed immediately by an equal volume of trypanosome suspension at the proper concentration. The adherent cells were incubated with trypanosomes and serum for 30 minutes at 37 C in a 5% CO_2 environment unless otherwise specified. After incubation with test reagents, Lab-Tek Chamber Slides containing adherent cells were washed extensively with PBS. The housing and gaskets were rapidly removed, the slides rinsed with PBS, and then dried with an air blower. Immediately upon drying, the slides were fixed with methanol (Baker Chemical C., Norristown, Pa.) for 60 seconds, air dried and then stained with a 4% solution of Giemsa stain (Original Azure Blend Type; Harleco, Gibbstown, NJ) for 30 minutes. Each of one hundred cells was recorded in one of four categories: 1) cells with only surface-bound trypanosome(s) 2) cells with internalized trypanosome(s) or portions of trypanosomes, 3) cells with both bound and internalized trypanosomes, and 4) cells with no identifiable trypanosome(s) or trypanosome debris. The identification of internalized trypanosomes was often difficult because of their partial digestion by the phagocytic cells.

The activity of antibody with respect to its ability to bind to macrophages prior to interaction with trypanosomes was investigated by incubation of the macrophage monolayer with 0.15 ml of medium and an equal volume of 1:10 anti-trypanosome serum. The antibody-monolayer combination was incubated for 30 or 45 minutes at 4C, 27C or 37C with intermittent shaking. The monolayers were vigorously washed to remove excess antibody and 0.3 ml of

trypanosomes at 40 x 10^6/ml were added, resulting in a final trypanosome-to-magrophage ratio of 80:1. (This assumes a 50% loss of cells during adherence and washing).

Electron microscopy. Peritoneal cells from C57BL/6J were removed according to standard procedure and resuspended at 3 x 10^6 cells/ml in RPMI 1640 with 20% FCS and antibiotics. A total of 1.5 ml of cells were plated onto plastic slides in Leighton tubes (Costar No. 3393, Cambridge, MA 02139) and incubated at 37C in a 5% CO_2 incubator. After 18h the cultures were washed with warm PBS to remove non-adherent cells. A total of 1.5 ml RPMI 1640 with FCS and antibiotics was then replaced in each tube and incubated 24h or 28h longer. Prior to assaying cell monolayers, the cultures were washed with PBS and replaced with 0.5 ml of a 1:5 dilution of the test rat or mouse (non-hyperimmune) serum, and an equal volume of trypanosomes adjusted with RPMI 1640 to final concentrations as noted in legends of figures. Trypanosomes and macrophages were incubated with normal or immune serum for various periods of time. These cells were washed several times in PBS, fixed in 4.75% glutaraldehyde solution with 4% sucrose and 0.1 M cacodylate buffer pH, and post-fixed in 1% osmium tetroxide. The preparations were then dehydrated and embedded in Epon 812. The resulting blocks were cut with a Porter-Blum MT-2 ultramicrotome with a Dupont diamond knife. Thin sections were mounted on 200 mesh nickel grids and stained with 1% uranyl acetate and lead citrate and examined with a JEOL 100 CX electron microscope.

For scanning electron microscopy, trypanosomes with macrophages and 1:5 normal or immune serum were washed in normal saline and fixed as described for transmission electron microscopy. The specimens were dehydrated in ethanol

and dried with liquid CO_2 in a Denton DCP-1 critical point dryer. Specimens were then coated with 100°A thick layer of gold pallidium and examined with a JEOL 100 CX scanning-transmission electron microscope at an accelerating voltage of 40 KV.

Results:

Requirement for immune serum for the phagocytosis of trypanosomes by murine adherent cells. Adherent cells incubated with trypanosomes in the absence of specific antibody did not bind any organisms during a 2h incubation period (Table I); in excess of 2h, some macrophage binding was observed. In contrast to the lack of early binding by macrophages in the presence of normal serum, there was substantial association of trypanosomes to macrophages in the presence of a immune serum within 30 minutes. There was a similarity in the extent of binding at 8h in both immune and normal serum, but it is assumed that in immune serum a number of the trypanosomes had already been digested and therefore could not be counted.

To determine the short-term time course of antibody dependent trypanosome binding to murine adherent cells, resident peritoneal macrophages were incubated in the presence of a 1:6 dilution of specific rat antiserum or normal serum for 0-120 minutes. Maximum attachment of trypanosomes occurred by 30-60 minutes with many fewer externally bound trypanosomes seen at 120 minutes (Fig 1). In the absence of serum, or in the presence of normal or medium rat serum, adherent cells were unable to bind trypanosomes during this incubation period.

Effect of trypanosome to adherent cell ratio on extent of binding. Aliquots of varying concentrations of trypanosomes were added to equal volumes of antiserum and incubated 30 minutes at 37C with 72h adherent cells. Trypanosome phagocyte ratios in excess of 125:1 resulted in conditions for attachment to levels

that were too dense to permit accurate microscopic quantitation of the phenomenon. Decreasing ratios through 30:1 facilitated counts, but still permitted greater than 95% of the macrophages to bind trypanosomes (Fig 2). At ratios of 16:1 and 8:1 trypanosome-to-adherent cell, there were intermediate levels of binding, with fewer than 15% of the cells binding trypanosomes at ratios below 8:1. Even when relatively low ratios of trypanosome adherent cell were used, e.g. 1:1, low levels of specific antiserum-mediated binding occurred.

Concentrations of trypanosomes which resulted in an effective final parasite:effector cell ratios of 75-100:1 gave consistent and replicable data and therefore were used throughout.

Effect on antiserum concentration on binding of trypanosomes by adherent cells. A serum titration experiment is illustrated in Fig. 2. In the presence of 1:10 rat antiserum, 100% of the macrophages bound trypanosomes; with a 1:20 dilution of antiserum, 50-80% binding resulted. Dilutions of this serum greater than 1:40 did not support association of trypanosomes and phagocytic cells.

Effect of prior antibody sensitization of cells or trypanosomes on binding. Macrophage monolayers were exposed to a 1:10 dilution of antiserum and incubated under varying conditions before they were washed to remove excess serum. Reaction mixtures of 0.3 ml of trypanosomes at 40 x 10^6/ml were added to the macrophage monolayers. After 30 or 60 minutes of incubation at 37C, the macrophages were washed to remove non-adherent trypanosomes, fixed and stained. In contrast with the 99% binding seen with control macrophages exposed simultaneously to antiserum and trypanosomes, (3%) which had been pretreated with antiserum bound trypanosomes (Table II).

When trypanosomes, rather than macrophages, were presensitized with antiserum, substantial uptake of these organisms by macrophages was observed. Equal volumes of trypanosomes at 75 x 10^6/ml were added to a 1:5 anti-trypanosome serum. The trypanosomes were incubated at varying temperatures for 45 min or 60 min at 37C. Under these conditions, greater than 90% of the macrophages bound trypanosomes (Table II).

Effect of abbreviated pre-incubation on ability of macrophages to bind trypanosomes. The following protocol (Fig 4) was carried out to determine whether shorter post-harvest incubations of resident cells from uninfected mice would influence the antiserum-dependent binding of trypanosomes. On day 1, peritoneal macrophages were prepared according to the standard protocol (culture A, Fig 4). On day 3, freshly harvested peritoneal cells were 1) incubated for a total of 6h (culture B1), washed of non-adherent cells and used immediately for the assay (B1* or 2) plated according to the standard 72h procedure (culture B2). The typical assay was performed by adding equal volumes of rat anti-trypanosome antibody (at a final concentration of 1:10) and trypanosomes (at an approximate 75:1 trypanosome-macrophage ratio, assuming 50% adherence) to both cultures A and B1 (A*, B1*). These trypanosome-antiserum mixtures and macrophages were incubated a total of 30 min at 37C in a 5% CO_2 incubator (Fig. 4). On day 6, culture B2 cells were assayed (B2*) to determine their ability to bind trypanosomes in the presence of immune serum. As seen in Fig 4, in the presence of antiserum, cells of all three cultures effectively bound trypanosomes regardless of the duration of preincubation. In fact, in this experiment a higher percentage of cells harvested 6h previously (culture B1), bound trypanosomes than those harvested 72h previously (cultures A and B2). It should be noted that these 72h adherent cells had lower

percentages in this experiment than was ordinarily encountered under similar conditions. In the absence of antiserum, there was negligible attachment of trypanosomes to adherent macrophages whether the monolayer had been in culture for 6h or for 72h (data not illustrated).

Interaction of trypanosomes with other types of adherent cells. To determine whether adherent peritoneal exudative cells were unique in their capacity to bind trypanosomes, BHK-21 (clone 15), Vero, P388D1, and normal peritoneal macrophages were cultured for 48h, 48, 120h, and 72h, respectively. The cells were washed thrice with warm PBS and mixtures of trypanosomes and non-hyperimmune serum were added. One tenth of a ml., immune or normal, rat serum was diluted 1:5 in RPMI 1640 and added to each chamber, followed immediately by an equal volume of trypanosomes at 112 x 10^6/ml. Adherent cell monolayers were incubated 30 minutes at 37C, washed with PBS and the slides fixed and stained. Of these four cell types, only P388D1 and normal murine peritoneal cells showed significant binding of trypanosomes, and only in the presence of immune serum (Table III).

Kinetics of specific antibody production in immunized mice. Mice were immunized with gamma-irradiated trypanosomes and bled at 3d and at 1, 2, and 4 weeks post-immunization. At 7 weeks they were boosted, and at weeks they were bled. Each serum sample was assayed at dilutions of 1:10 to 1:160 to determine its ability to mediate attachment of trypanosomes to 72h murine macrophage monolayers. Fig. 5 is a representation of the percentages of macrophage-associated trypanosomes in the presence of a 1:160 dilution of mouse antisera obtained over a period of 8 weeks post-immunization. This antiserum dilution provided the greatest discrimination as to the extent of antiserum-mediated attachment of trypanosomes to macrophages.

As early as 3d post-injection, mice generated antiserum which was able to mediate some association of trypanosomes with macrophages (Fig 5). The ability of the serum to mediate binding/ingestion peaked at 1 week, at which time more than 85% of the macrophages bound trypanosomes. The percentage of mononuclear cells which displayed associated trypanosomes declined to 24% by the 4th week. Following a booster injection at 7 weeks, the antibody potency rose again to heights that mediated association of trypanosomes to greater than 95% of the population of macrophages, even at a titer of 1.160 (Fig. 5).

Complement enhancement of phagocytosis in the presence of limiting dilutions of antiserum. The data suggests that complement enhances the binding of trypanosomes to macrophages in the presence of limiting dilutions of antiserum. In a representative experiment (Fig 6), immune serum which had been heat-treated to inactivate complement resulted in progressively lower percentages of trypanosomes binding to macrophages as the antiserum was diluted 1:10 to 1:40. When 25% complement was added to these samples, significant restoration of high numbers of macrophages binding trypanosome were observed in each case (Fig 6).

Lack of enhanced uptake by cells of BCG-treated mice. BCG (Mycobacterium bovis) treatment of mice induces macrophages to exhibit many of the characteristics of activated macrophages (41). BCG-induced cells were compared with normal, non-induced macrophages for their ability to take up trypanosomes in the presence of decreasing concentrations of heat-inactivated rat antiserum. In the presence of 1.10 dilution of immune serum, over 90% of both normal resident cells and BCG-induced cells bound trypanosomes (Table IV). Further dilutions of antiserum resulted in a progressive decline in trypanosome uptake, but no pattern emerged which suggested that either of the cell populations was more efficient. In the presence of normal rat serum, neither cell population was capable of binding trypanosomes.

Electron microscopy. In the presence of antiserum, many trypanosomes could be demonstrated within macrophages by electron microscopy (Fig 7). These parasites were found within vacuoles, often with several parasites present within an individual vacuole (Fig 8). Incubation of macrophages with trypanosomes and immune serum for up to 90 min. resulted in no morphological changes in the rapidly interiorized trypanosomes; however, by 120 min the parasites had become electron-dense (Figs 9, 10). Scanning electron microscopy (SEM) in the presence of immune serum showed many trypanosomes adhered to macrophage surfaces, and on occasion the parasite's flagellum extended into the macrophage cytoplasm (Figs 11, 12). In contrast, in the presence of normal serum or medium, incubation of macrophages and trypanosomes for even 120 min revealed little association between parasites and adherent macrophages (Fig 13). With longer incubation intervals SEM demonstrated some internalization of trypanosomes, with the organisms within unit membranes. No morphological changes were seen in these parasites.

DISCUSSION

In this report we confirm and extend previous findings (7,8,14,30,53,54, 55,58,60) that macrophages from noninfected mice, rats, or rabbits, can bind and ingest sensitized African trypanosomes in the presence of specific antibodies. We present evidence that the complement system participates in the association of macrophages and Trypanosoma rhodesiense.

Previous investigators (50), using radiolabeled parasites, described phagocytosis of Trypanosoma brucei in the presence of specific antiserum, by peritoneal macrophages derived from deer mice (Peromyscus maniculatus) infected with T. brucei. Cells from uninfected mice did not function in this reaction. The authors interpreted this as evidence that activation of macrophages by prior infection of deer mice with trypanosomes was a prerequisite for in vitro phagocytosis.

The data presented in this report, as well as the studies of other investigators, demonstrated that macrophages from uninfected mice (Mus musculus) (7,30) or rats (Rattus norvegicus) (55) were able to efficiently bind trypanosomes providing that specific antibody was present. Differences in the functional capabilities of the macrophages, due to either experimental or natural variables, may account for the discrepancies found among these studies. For example, the pre-incubation time of adherent cells prior to addition of trypanosomes is a variable which potentially could be influential. It has been reported (5) that cultivation of murine macrophages for 48h-72h (such as was performed in the present study), may result in morphological changes and increases biological activity such as uptake of antibody-coated erythrocytes. To determine whether shorter macrophage pre-incubation times would result in different degrees of uptake, we incubated resident murine macrophages for the same period (6h) as resident deer mouse macrophages were incubated in the study of Stevens and Moulton (50).

As was reported in this study, adherent cells harvested both 6h and 72h previously bound trypanosomes equally. Also, activated cells obtained by BCG-injection of mice resulted in macrophage populations with levels of trypanosome attachment similar to those observed with macrophages from untreated mice.

The finding of Stevens and Moulton that trypanosome uptake occurred only with macrophages from infected animals, but not normal animals, is in conflict with the data reported in this study as well as others (30,53,54,55). It is possible that these disagreements are dependent upon differences in the animals used. For example, P. miniculatus belongs to the more primitive Cricetidae family, while M. musculus belongs to the Muridae family. Ample precedence for species, e.g. human vs. mouse (25), and strain, e.g. C3H/HeN vs. C3H/HeJ. (Dr. S. Vogel, personal communication)(43), differences exist in other macrophage/target systems. Other explanations can be envisioned; the possibility that the murine macrophages have been activated by a cryptic infection, however unlikely, also cannot be absolutely ruled out.

The contribution of the complement system in the association of African trypanosome with macrophages has not been resolved. A number of investigators have reported that the phagocytosis of African trypanosomes by mouse (7,8), rat (14,55), or rabbit (58) macrophages occurs in the absence of complement. Others are of the opinion that the presence of complement can facilitate phagocytosis (28,50). These differences in interpretation are explicable in terms of the current understanding of the role of complement in phagocytosis in other systems.

Phagocytosis of sensitized particles can be separated into two discrete phases: 1) adherence of the particle to the surface of the phagocytic cell, and 2) internalization, (40,44). These phenomena are regulated by at least two sets of receptors; one set is specific for the activated third component of complement (12) and the other for the Fc domain of IgG (4).

In the absence of immunoglobulin, red cells coated with complement components will bind to macrophages, but not be ingested (2, 31). In contrast, high concentrations of immunoglobulin can mediate both attachment and ingestion in the absence of complement (11, 35). Complement may, however, act in synergy with immunoglobulin. For example, when coated with complement, 10^2 fewer IgG molecules are necessary to promote internalization of IgG-sensitized particles (12). This experimental demonstration of synergy provides an explanation for the reversal by complement of depressed levels of attachment and binding seen in the presence of low titers of IgG (11, 35, 61).

In contrast with the complement-independent nature of IgG when mediating phagocytosis under certain conditions, complement is considered to be an absolute requirement for IgM intercession (11, 62). This immunoglobulin must complex with its antigen, fix complement, and only through the activated portion of the third component of complement, can it associate with the complement receptor of the macrophage (5, 59).

In the present study, under stated conditions, antibody-dependent binding of trypanosomes to macrophages was consistently observed in the presence of 1:5-1:10 antiserum depleted of complement by heating at 53C for 90 min. However, in agreement with the patterns seen in the above studies, when lower, less effective concentrations of antiserum were utilized, the addition of complement profoundly amplified the association of trypanosomes and macrophages.

This evidence suggests that Fc receptors, either independently or with the cooperation of complement receptors, are involved in the binding of trypanosomes to macrophage surfaces. This interpretation is further supported by our finding that specific antibody-mediated attachment of trypanosomes to P388D1 cells occurred with only negligible association of trypanosomes to Vero or BHK-21 cells.

Figure Legends

Fig. 1 The effect of incubation time on the binding of trypanosomes macrophages in the presence of 1:6 dilutions of immune (●) or normal (△) serum.

Fig. 2 Effect of decreasing trypanosome to adherent cell ration on the percentage of macrophages with bound trypanosomes.

Fig. 3 Trypanosome attachment to macrophages in the presence of decreasing amounts of anti-trypanosomes serum.

Fig. 4 Effect of shortened initial incubation time on binding of trypanosomes by macrophages. Cultures B1 and B2 are from the same pool of cells. *Culture assayed in standard manner with antibody and trypanosome. Mean $\pm$S. E. M. of quadruplicate samples.

Fig. 6 Effect of addition of 25% complement to decreasing concentrations of heat-inactivated immune serum (DIX) and their ability to mediate binding.

Fig. 7 Electron micrograph of trypanosomes and macrophages incubated 15 min in the presence of mouse antiserum. Note numerous trypanosomes (arrow) within the macrophages. x 7,000.

Fig. 8 Higher power electron micrograph of a macrophage incubated with trypanosomes in the presence of antiserum. A number of trypanosomes (arrow) are present within individual vacuoles. x17,000.

Fig. 9 Electron micrograph of a macrophage incubated with trypanosomes and mouse antiserum for 120 min. Note degenerated trypanosomes (arrow) within the macrophage. x8,000.

Fig. 10 Higher power micrograph of degenerating trypanosomes after 120 min incubation in the presence of mouse antiserum. A flagellum (arrow) is still identifiable. x18,000.

Fig. 11 Scanning electron micrograph of a macrophage incubated with trypanosomes in the presence of antiserum for 15 min. Two trypanosomes are attached to the macrophage. x9,000.

Fig. 12 High power scanning electron micrograph of a macrophage incubated with trypanosomes in the presence of antiserum. A flagellum of the parasite is enveloped by the macrophage (arrow). x40,000.

Fig. 13 Electron micrograph of trypanosomes and macrophages incubated in the presence of media for 120 min. Please contrast with Fig. 7 (15 min incubation with immune serum) and Fig 9 (120 min with immune serum). Arrow indicates trypanosome. x7,000.

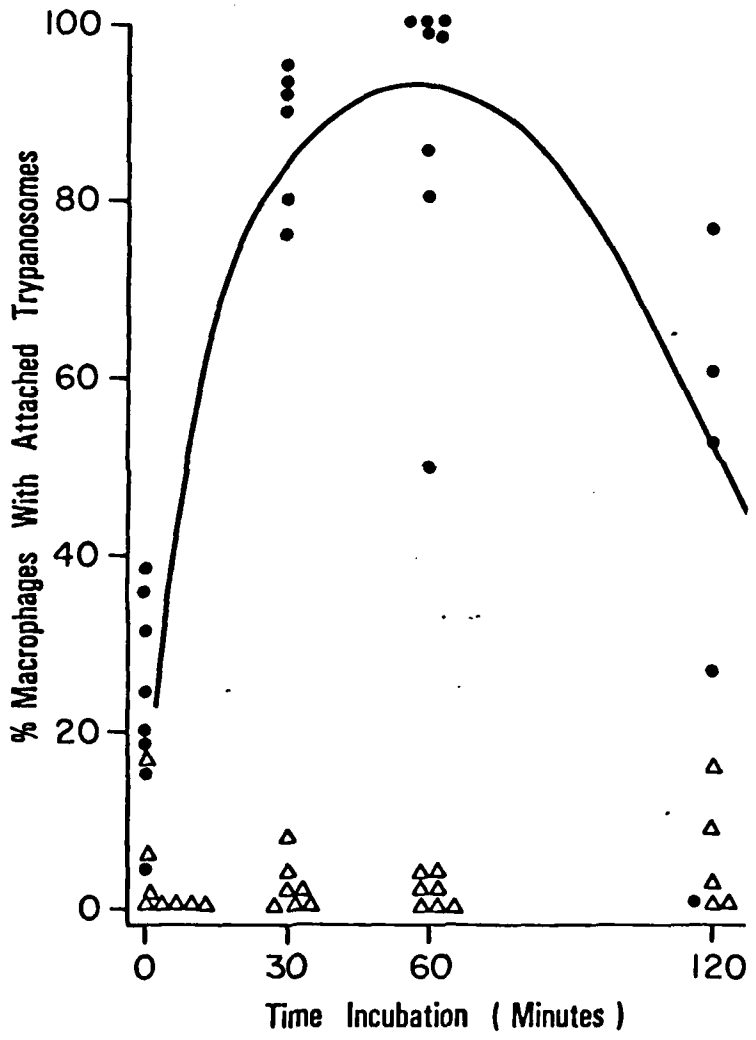

100
80
60
40
20
0
% Macrophages With Attached Trypanosomes
0
30
60
120
Time Incubation (Minutes)

Fig 1

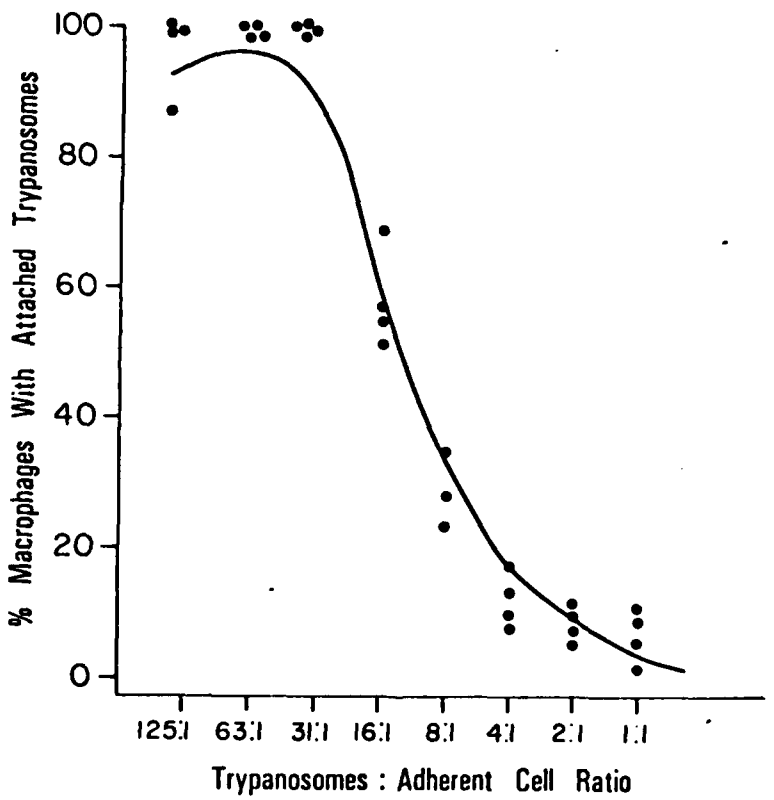

% Macrophages With Attached Trypanosomes
100
80
60
40
20
0
125:1
63:1
31:1
16:1
8:1
4:1
2:1
1:1
Trypanosomes : Adherent Cell Ratio

Fig 2

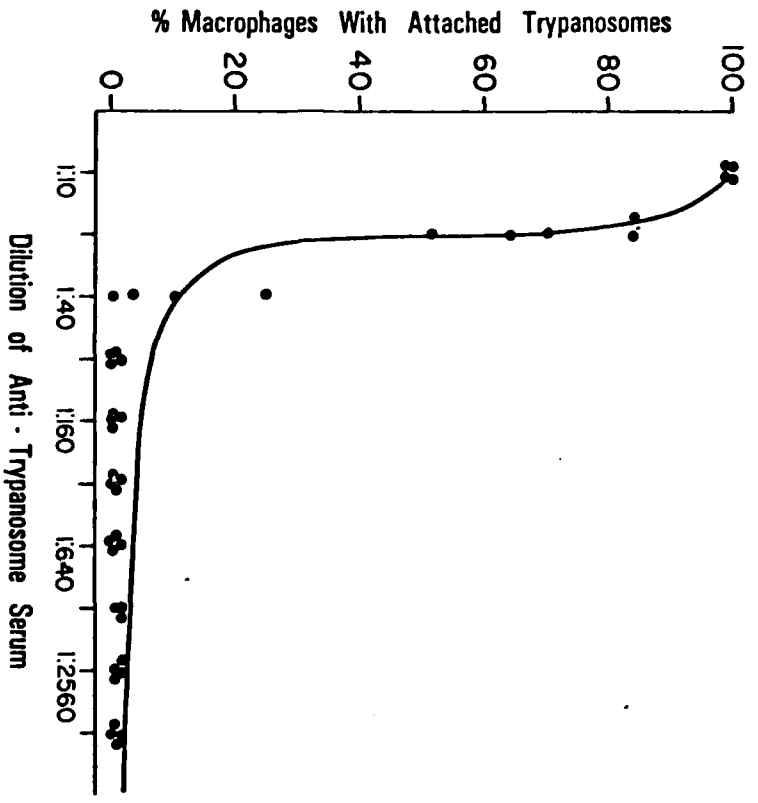

% Macrophages With Attached Trypanosomes
0
20
40
60
80
100
Dilution of Anti - Trypanosome Serum
1:10
1:40
1:160
1:640
1:2560

Fig 3

Day of Initiation of Culture and / or Assay	Source of PEC's	% of Macrophages with Associated Trypanosomes
1	Mouse A → Culture A	
3	Mouse B → Culture A *	34.3 ± 6.4[a]
	→ Culture B1*	70.3 ± 3.0
	→ Culture B2	
6	Culture B2*	45.3 ± 9.5

Fig 4

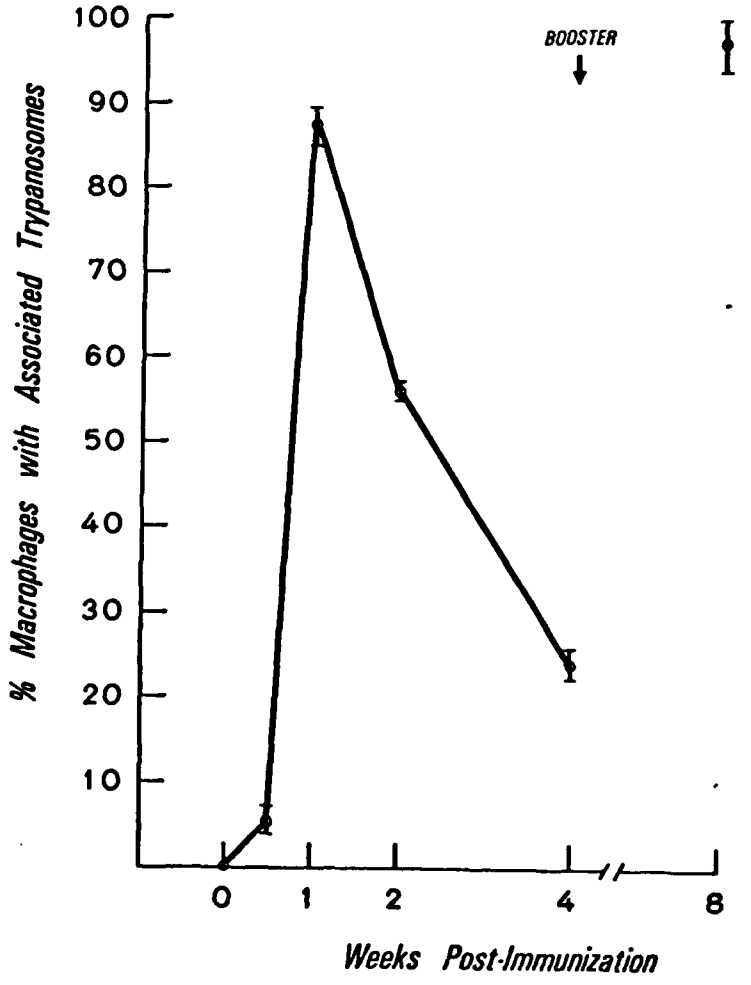

100
90
80
70
60
50
40
30
20
10
% Macrophages with Associated Trypanosomes
BOOSTER
0
1
2
4
8
Weeks Post-Immunization

Fig 5

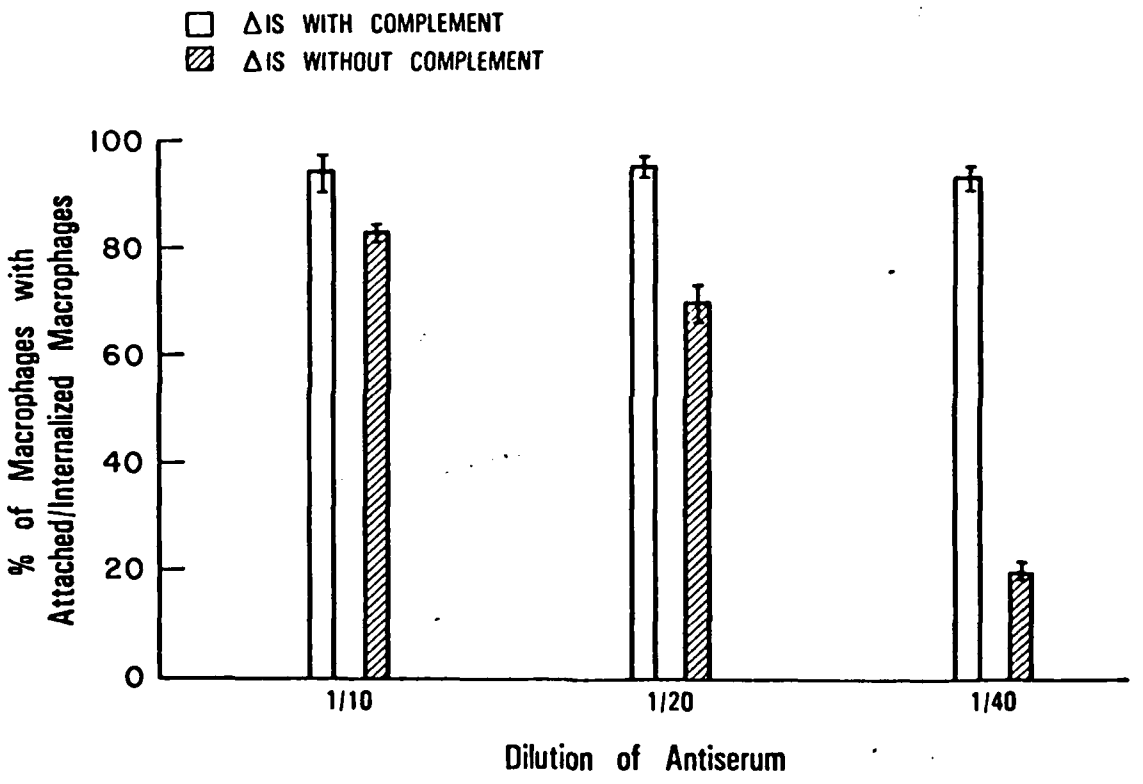
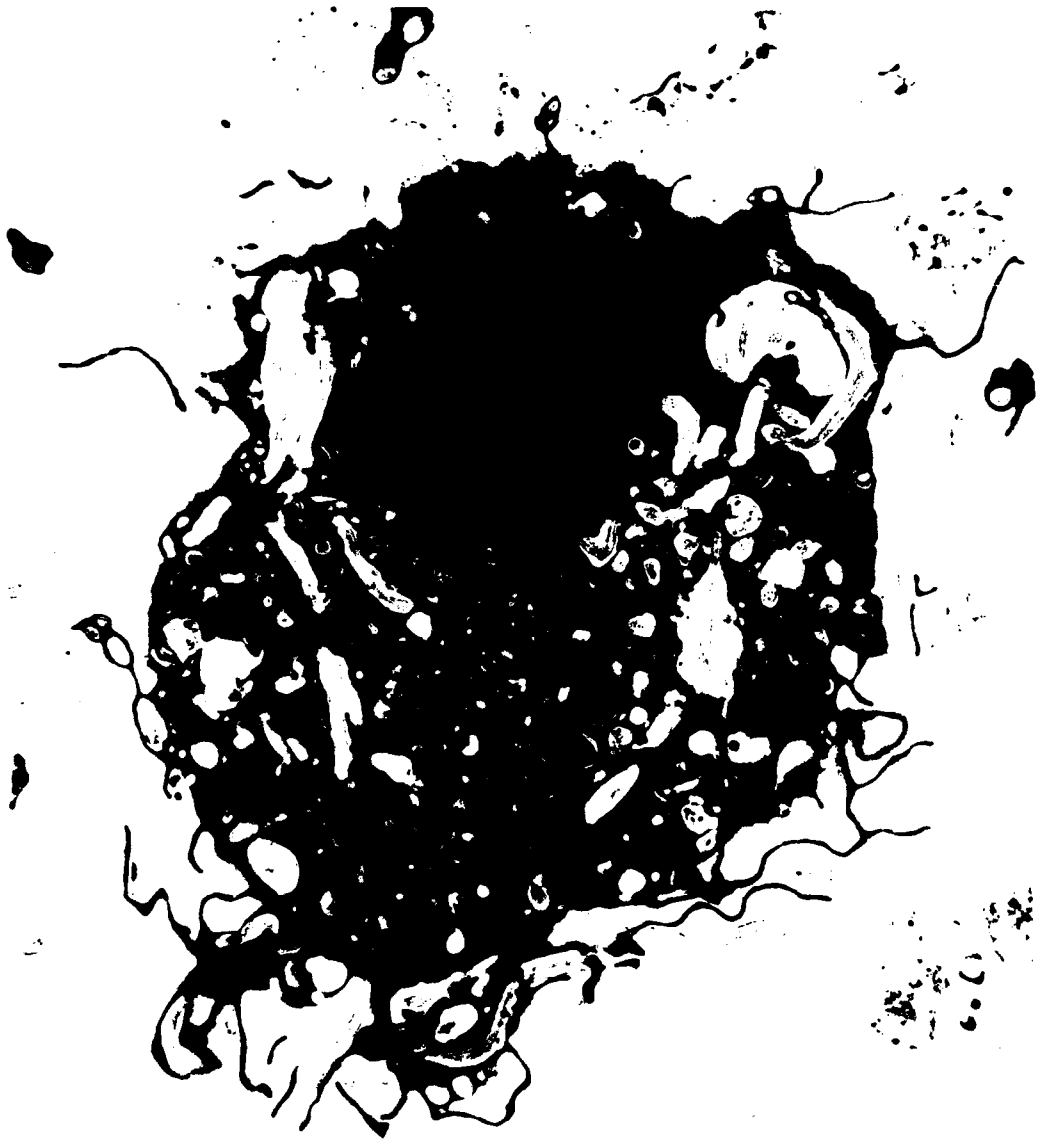

□ ΔIS WITH COMPLEMENT
▨ ΔIS WITHOUT COMPLEMENT
% of Macrophages with Attached/Internalized Macrophages
100
80
60
40
20
0
1/10
1/20
1/40
Dilution of Antiserum

Fig 6



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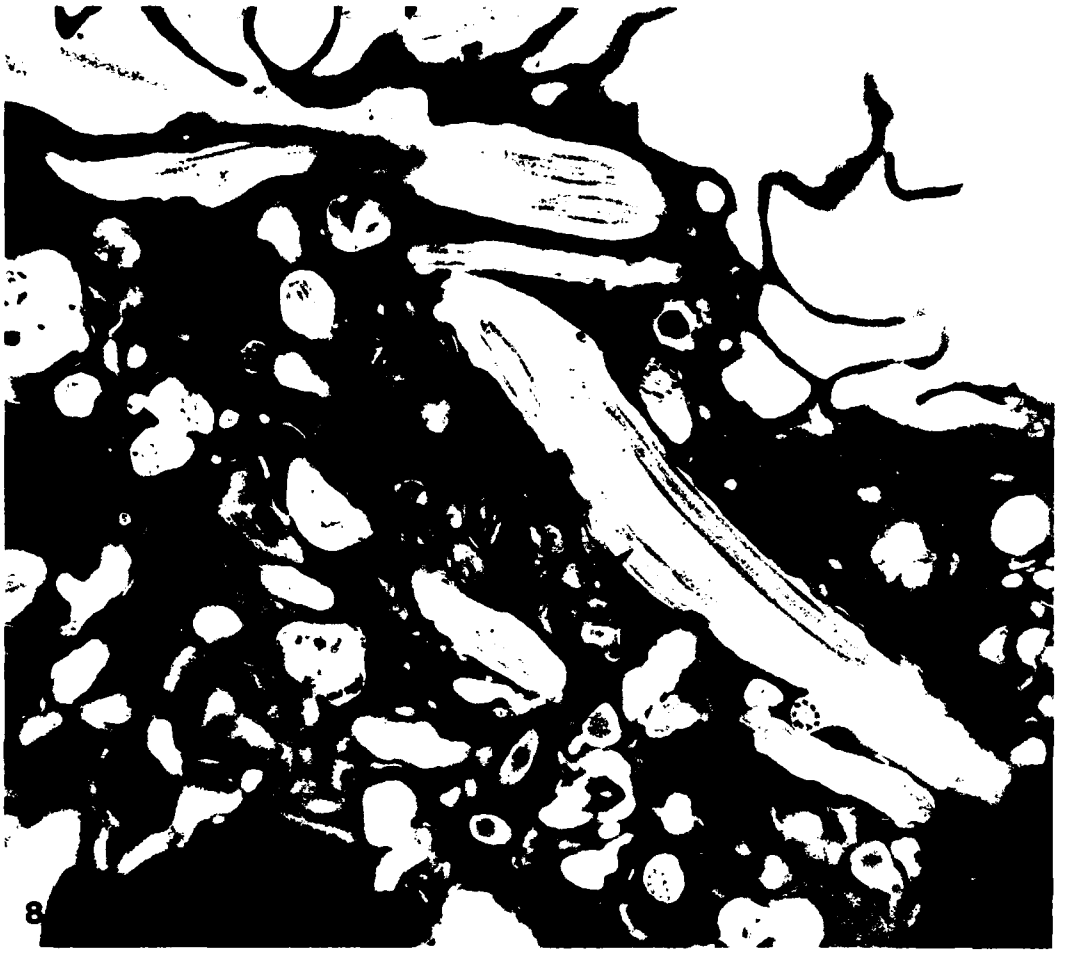


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